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## Diploid male production correlates with genetic diversity in the parasitoid wasp *Venturia canescens*: a genetic approach with new microsatellite markers

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### Keywords

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## Introduction

Sex determination takes on many different forms in animals (Beukeboom and Perrin 2014). In insects of the order Hymenoptera, haplodiploidy is the rule: Males develop from unfertilized eggs and are haploid, whereas females develop from fertilized eggs and are diploid. In numerous species including sawflies, pollinating bees, social and solitary wasps, ants, and parasitoids, the underpinning genetic mechanism of sex determination relies on the complementarity of alleles at a single gene (Whiting 1943; van Wilgenburg et al. 2006; Heimpel and de Boer 2008). This gene – *csd*, for complementary sex determiner – has been described in the honeybee and comes from the duplication of the *transformer* gene (Geuverink and Beukeboom 2014).

## Abstract

Sex determination is ruled by haplodiploidy in Hymenoptera, with haploid males arising from unfertilized eggs and diploid females from fertilized eggs. However, diploid males with null fitness are produced under complementary sex determination (CSD), when individuals are homozygous for this locus. Diploid males are expected to be more frequent in genetically eroded populations (such as islands and captive populations), as genetic diversity at the *csd* locus should be low. However, only a few studies have focused on the relation between population size, genetic diversity, and the proportion of diploid males in the field. Here, we developed new microsatellite markers in order to assess and compare genetic diversity and diploid male proportion (DMP) in populations from three distinct habitat types – mainland, island, or captive –, in the parasitoid wasp *Venturia canescens*. Eroded genetic diversity and higher DMP were found in island and captive populations, and habitat type had large effect on genetic diversity. Therefore, DMP reflects the decreasing genetic diversity in small and isolated populations. Thus, Hymenopteran populations can be at high extinction risk due to habitat destruction or fragmentation.

The *csd* gene is probably responsible for arrhenotoky in all species of the order Hymenoptera (Beye et al. 2003; Asplen et al. 2009; Schmieder et al. 2012). Under single-locus complementary sex determination (sl-CSD), unfertilized eggs are hemizygous at the *csd* locus and develop normally into haploid males (Whiting 1943). Among the fertilized eggs, only heterozygous individuals develop into females; homozygosity at the *csd* locus yields diploid males that are generally, but not always unviable or sterile (Cowan and Stahlhut 2004; Heimpel and de Boer 2008; Harpur et al. 2013). sl-CSD is the mechanism of sex determination for which empirical evidence prevails in Hymenoptera (Cook 1993; van Wilgenburg et al. 2006; Heimpel and de Boer 2008), but other mechanisms such as multilocus CSD or genomic imprinting also occur (Verhulst et al. 2010).

At the population level, sl-CSD causes higher sensitivity to losses of genetic diversity. A decrease in allelic richness at the *csd* locus and a subsequent increase in homozygosity are expected to result in more diploid males and, in turn, lower population growth rate. Theoretical models predict that sl-CSD is at the core of a “diploid male vortex,” a feedback between demographic and genetic processes that potentially drives bottlenecked populations toward extinction (Zayed and Packer 2005; Hein *et al.* 2009; Fauvergue *et al.* 2015; Bompard *et al.* submitted). However, diploid males represent such fitness cost that various individual behaviors have evolved to limit their production. Dispersal limits inbreeding and the consequent diploid male production (Ruf *et al.* 2011), and also allows the recruitment of new *csd* alleles, so that well-connected populations are expected to produce fewer diploid males (Hein *et al.* 2009). Some Hymenoptera with sl-CSD have undifferentiated populations beyond a 100-km scale (Estoup *et al.* 1996; Zimmermann *et al.* 2011), suggesting high dispersal abilities. Mate choice can also influence the production of diploid males with a reduction expected in case of inbreeding avoidance (Whitehorn *et al.* 2009; Metzger *et al.* 2010a; Thiel *et al.* 2013). Such behaviors may mitigate the diploid male vortex (Hein *et al.* 2009).

The central assumption of CSD population models is that small, bottlenecked, or isolated populations have a low genetic diversity, including diversity at the *csd* locus, and consequently a high proportion of diploid males. To date, however, empirical support to this assumption is scarce. Only rare field data on fire ants (Ross *et al.* 1993), bumblebees (Darvill *et al.* 2006, 2012), or paper wasps (Tsuchida *et al.* 2014) suggest a relationship between population size, genetic diversity, and the proportion of diploid males. These studies have all relied on genetic markers to estimate within-population genetic diversity, gene flow between distinct populations, mating structure, and, in some cases, the proportion of diploid males.

Evidence for diploid males in natural populations mainly concerns social Hymenoptera. In these species, the dramatic bias in sex ratio toward females renders estimations of the proportion of diploid males very problematic. In parasitoid wasps, the proportion of diploid males was assessed in only three related species: *Cotesia glomerata* (Ruf *et al.* 2013), *Cotesia rubecula* (de Boer *et al.* 2012), and *Cotesia vestalis* (de Boer *et al.* 2015). Parasitoid populations often experience small population size, for example as a consequence of cyclic dynamics (Hassell 2000) or biological control introductions (Hopper and Roush 1993). Given the key role that parasitoid wasps play by controlling the populations of herbivorous insects in natural ecosystems and agrosystems (Shaw and Hochberg 2001), the scarcity of field estimations on the occurrence of diploid males in the field is surprising.

*Venturia canescens* G. (Hymenoptera: Ichneumonidae) is a parasitoid wasp with sl-CSD (Beukeboom 2001). This species is a model organism commonly used in laboratory studies on behavior, physiology, and life history traits (Harvey *et al.* 2001; Desouhant *et al.* 2005; Pelosse *et al.* 2007). Two reproduction modes are known in this species (Schneider *et al.* 2002): thelytoky (asexual, parthenogenesis) and arrhenotoky (sexual reproduction under haplodiploidy). In arrhenotokous *V. canescens*, diploid males are fully viable and able to mate. However, they are sterile, and females with whom they mate produce only sons, similar to virgin females (Fauvergue *et al.* 2015). The prevalence of diploid males in natural or captive *V. canescens* populations has not been estimated yet, and little is known about the genetic structure of natural populations. So far, the rare population genetic studies on *V. canescens* showed the absence of genetic structure at the geographic scale of the French Riviera (Schneider *et al.* 2002; Mateo Leach *et al.* 2012).

We developed this study with a threefold objective: (1) develop microsatellite markers in order to assess male ploidy and conduct population genetic studies on arrhenotokous *V. canescens*; (2) estimate the proportion of diploid males in several populations; and (3) estimate the genetic diversity of *V. canescens* populations under various conditions of population size and isolation. For this, we compared mainland, island, and captive populations, with the expectation of a lower genetic diversity and a higher proportion of diploid males in captive populations compared to insular populations and in insular populations compared to mainland populations.

## Materials and Methods

### Sampling

Female *V. canescens* were collected during summer 2010 in two natural populations located in southern France near Nice and Valence (Table 1). To attract parasitoid females, open cups containing a mixture of host-rearing medium (semolina) and larvae of the host *Ephesia kuehniella* (Lepidoptera: Pyralidae) were hung in trees. Infested semolina is impregnated with host kairomones and is very attractive for *V. canescens* females (Corbet 1971; Metzger *et al.* 2008). Females visiting the traps to oviposit were collected, brought into the laboratory, allowed to lay eggs for 2 days on a host patch, and then killed and preserved in 96% ethanol. The reproductive mode of captured females was tested by checking the presence of males among offspring. Only arrhenotokous (sexual) females were used in the following analysis.

In addition, males were searched in eighteen locations of the Mediterranean Basin between 2011 and 2013 during late summer and autumn. Five sites were located on

**Table 1.** Studied populations: locality, habitat type (mainland, island, and captive populations), geographic coordinates, host plant (Car, Fig, Wal, Pom, Che, Pea, Haz, Cit, and Oli being respectively carob, fig, walnut, pomegranate, cherry, peach, hazelnut, citrus, and olive trees), year of sampling, and year of foundation between parentheses for captive populations, with the corresponding number of males or females sampled. See Figure 1 for a map of localities.

Populations						Sampling		
Habitat type	Country	Locality	Name	Geographic coordinates	Host plant	Date	Number of males	Number of females
Mainland	France	Valence	Val10	44°58'21"N 4°55'39"E	Che/Pea/Haz	2010	0	37
		Nice	Nice10	43°41'23"N 7°18'6"E	Car	2010	0	44
		Nice	Nice11	43°41'23"N 7°18'6"E	Car	2011	190	0
		Nice	Nice13	43°41'23"N 7°18'6"E	Car	2013	90	21
		Solliès	Sol13	43°10'58"N 6°2'55"E	Fig/Wal/ Pom/Che	2013	16	0
	Spain	Vila-Seca	VS13	41°7'34"N 1°8'07"E	Car	2013	58	2
		Vinyols	Vy13	41°6'12"N 1°2'26"E	Car/Oli	2013	33	2
Island	France	Corsica	NA <sup>1</sup>			2013	0	0
		Porquerolles	NA <sup>1</sup>			2013	0	0
	Spain	Mallorca	Mlc12	39°47'58"N 2°57'53"E	Car	2012	21	0
		Mallorca	Mlc13	39°47'58"N 2°57'53"E	Car	2013	38	3
	Italy	Sicily	NA <sup>1</sup>			2012	0	0
	Greece	Crete	Cre12	35°11'39"N 25°2'09"E	Pom/Fig/Cit/Oli	2012	0	1
		Crete	Cre13	35°11'39"N 25°2'09"E	Pom/Fig/Cit/Oli	2013	0	1
	Malta	Gozo	Goz12	36°3'34"N 14°16'34"E	Car	2012	0	2
	Cyprus	Cyprus	Cyp12	34°39'5"N 33°0'17"E	Car	2012	0	1
		Cyprus	NA	34°39'5"N 33°0'17"E	Car	2013	0	0
Captive	France	Nice	CapNiceA	43°41'23"N 7°18'6"E	Car	2013 <sup>2</sup> (2011)	50	0
		Nice	CapNiceB	43°41'23"N 7°18'6"E	Car	2013 <sup>3</sup> (2011)	31	0
		Valence	CapVal	44°58'21"N 4°55'39"E	Che/Pea/Haz	2013 (2013)	50	0
	Israel	Tel-Aviv	CapIsr	Not Available	Not Available	2013 (2011)	50	0

<sup>1</sup>Several sites were searched in these locations, but no wasps were found.

<sup>2</sup>Sampled in July 2013.

<sup>3</sup>Sampled in October 2013.

mainland in Nice (France, 2011 and 2013), Solliès (France, 2013), and Vinyols and Vila-Seca (Spain, 2013). In 2012, we searched *V. canescens* males in five sites located on islands: Mallorca (Spain), Gozo (Malta), Crete (Greece), Sicily (Italy), and Cyprus. Several sites per island were sampled. In 2013, the same protocol was followed to capture males on French islands (Porquerolles and Corsica), in Mallorca (Spain), Cyprus, and in Crete (Greece) (Table 1

and Fig. 1). We selected field sites with at least 10 individual host plants (i.e., carob, fig, pomegranate, citrus, palm, or walnut trees; Salt 1976). On each site, ten male traps were evenly distributed. They were hung in host trees at 50–150 cm from the ground. Traps were checked after 48 h. If one *V. canescens* individual was caught, 20–40 traps were added on the site. *V. canescens* males are attracted by the synergy of pheromones produced by females and

kairomones from their hosts (Metzger et al. 2010b). Traps baited with extracts from these semiochemicals were designed to attract and capture males. A trap consisted of a 125 mm × 200 mm yellow sticky sheet, in the center of which was hung a vial containing the extract. To prepare the extract, 100 g of host-rearing medium containing larvae was immersed during 1 h in 200 mL of hexane. The solution was filtered, and one female was soaked in 100 µL for 3 h. The female was then removed, and the extract was stored at −20°C. A few hours before being used in the field, the extract was evaporated by heating the vial on a hot plate (this step was skipped for the 2013 campaign, after it was shown that evaporation occurred within a few hours in the field). As for females, traps were hung within host trees, and all *V. canescens* males that stuck on the traps were collected and preserved individually in 96% ethanol.

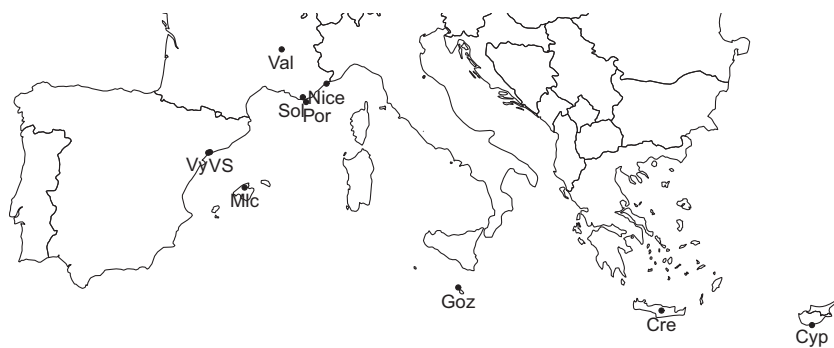
In parallel, we collected males in three captive (laboratory mass-reared) populations differing in their history and geographic origin (Table 1): (1) a 25–30-generation-old population founded with about 120 females from Nice, (2) a 10–15-generation-old population founded with about 100 females from Valence, and (3) a 15–20-generation-old population founded with 11 females from Israel. Hence, these captive populations experienced different intensities of founding effects, genetic drift, and inbreeding. The three populations were maintained with a standard protocol on larvae of the host *E. kuehniella* obtained by rearing 50 mg of *E. kuehniella* eggs (about 2000 eggs) provided by Biotop (Livron-sur-Drôme, France) in plastic boxes (8 × 12 × 25 cm) set with 250 g of wheat semolina. Each week, three boxes containing second to fifth instar host larvae were inoculated with 50 males and 50 females of *V. canescens* emerging from all available (about six) rearing boxes.

## Development of microsatellite genetic markers

Using a DNeasy Tissue Kit from Qiagen (QIAGEN, Hilden, Germany), DNA was extracted from a single 1.5-mL

tube containing a pool of 20 adult females of *V. canescens* recently collected from the Nice population and kept in the laboratory for a few generations. The obtained DNA solution was enriched in microsatellites and pyrosequenced by the company Genoscreen (Lille, France), following the protocol described in Malausa et al. (2011). Using the iQDD program (Meglec et al. 2010), primer pairs were designed for 675 microsatellite loci (Malausa et al. 2011), among which 124 were screened in monoplex PCR on DNA of one thelytokous and six arrhenotokous *V. canescens* females from three populations: Valence, Nice, and Antibes (geographic coordinates: 43°33'51"N, 7°7'28"E). Six primer pairs designed by Mateo Leach et al. (2012) were also tested following the same protocol.

The DNA extracts used for these monoplex PCR were obtained using the DNeasy Tissue Kit of QIAGEN. Each monoplex PCR contained 2 µL of DNA, 5 µL of 2X QIAGEN Multiplex PCR Master Mix, and 0.2 µmol/L of each primer. The total volume was adjusted to 10 µL with ultrapure water. PCR was performed as follows: a step of denaturation at 95°C during 15 min, followed by 25 cycles of 30 sec of denaturation at 94°C, 90 sec of annealing at 58°C and 60 sec of extension at 72°C, and a final extension step of 30 min at 60°C. PCR products were separated on a 2% agarose gel stained with ethidium bromide to detect amplification of DNA fragments. For 37 successfully amplified microsatellite loci, forward primers labeled with one of four fluorochromes (Applied Biosystems, Carlsbad, CA) were used in a monoplex PCR with the DNA samples and conditions previously described. Two microliters of PCR product was added to 8.75 µL of Hi-Di formamide and 0.25 µL of GeneScan500 Liz size standard (Applied Biosystems). The mix was loaded on an ABI 3130XL genetic analyzer (Applied Biosystems), and alleles were scored using Gene Marker<sup>®</sup> version 1.75 (SoftGenetics, State College, PA). Monomorphic markers were discarded, and the remaining markers were combined according to their size and fluorochrome in several multiplex PCRs that were tested on the same seven individuals and in the same PCR conditions as previously



**Figure 1.** Location of field sampling. Cre, Cyp, Goz, Mlc, Nice, Sol, Val, VS, and Vy are respectively acronyms for Crete, Cyprus, Gozo, Mallorca, Nice, Solliès, Valence, Vila-Seca, and Vinyols. As no wasps were found in Porquerolles, Corsica, and Sicily, these locations are not presented here.



described. Finally, we selected 19 microsatellite loci amplified using two multiplex PCRs (Table 2).

### Assessment of male ploidy

We used the genetic markers from the multiplex PCR I (Table 2) to detect diploid males: If a male is found heterozygous for at least one locus, it is considered diploid (Zhou *et al.* 2006; Armitage *et al.* 2010; Souza *et al.* 2010). However, if a diploid male is homozygous for all loci, it will be falsely scored haploid. To estimate the power of the developed microsatellite markers to correctly assess male ploidy, we calculated the probability that a diploid male is homozygous at all the 10 loci of the multiplex and, hence, falsely considered haploid. For this, we first calculated the probability that a diploid male produced by a brother–sister pair is homozygous for all 10 loci of the multiplex PCR I (see Annex). In parallel, we compared ploidy assessed on the same individuals via microsatellite genotyping and flow cytometry. Measure of male ploidy by flow cytometry is based on DNA quantity in cell nuclei (haploid nuclei being expected to have twice less DNA than diploid nuclei). To produce enough diploid males for analysis, brother–sister pairs were formed with individuals from a captive population. From the offspring, 39 males were collected and killed: The thorax was used for genetic analyses and the head for flow cytometry.

We performed flow cytometry analyses as described in de Boer *et al.* (2007). Only the head of insects was used for flow cytometry because endoreduplication doubles the quantity of DNA in cells of other parts of the body of haploid males, making haploid and diploid nuclei undistinguishable (Aron *et al.* 2005). To isolate cell nuclei, each insect head was crushed in 0.5 mL of Galbraith buffer by turning the B pestle 20 times in a Dounce tissue grinder maintained on ice. The homogenate was filtered through a 40- $\mu$ m cell strainer cap. Cell nuclei were stained with 20  $\mu$ L propidium iodide at 1 mg/mL. Nuclei were analyzed on a LSRII Fortessa flow cytometer (BD Biosciences, San Jose, CA) with an excitation wavelength of 561 nm. Using FACSDiva Version 6.1.3 (BD Biosciences), we analyzed 10,000 nuclei per sample in a region that excluded doublets and debris. Flow cytometric DNA histograms of diploid females were used as reference to identify diploid males. The ploidy of each captured male was deduced from its genotype at microsatellite markers from multiplex PCR I. A male was considered diploid if it was heterozygous at least at one locus.

### Population genetics

#### Validation of microsatellites on female wasps

To be used in population genetic studies, each marker must have several detectable alleles and must not be

linked with any other marker or locus under selection. We therefore measured genetic polymorphism, estimated frequencies of null alleles, and test for departures from Hardy–Weinberg (HW) equilibrium and linkage disequilibrium on the two mainland populations from Valence and Nice where enough females were captured in 2010 (Table 1). DNA of each captured female was extracted with the PrepGem Insects kit (ZyGEM, Hamilton, New Zealand). DNA extraction, PCR, and genotyping were performed as previously described. The GENEPOP software version 4.3 (Rousset 2008) was used to calculate the number of alleles, the expected and observed heterozygosity, and to test for HW equilibrium and linkage disequilibrium between loci. We estimated null allele frequencies with the FreeNA program (Chapuis and Estoup 2007).

#### Population structure and male ploidy

For all males captured, DNA was extracted, PCR were performed with multiplex I, and genotypes were analyzed as previously described. Only males with at least eight loci genotyped were used for the analysis. Coexistence of male haploidy and diploidy impedes the estimation of classical population genetic statistics such as observed and expected heterozygosity or Wright's *F* statistics. We therefore computed a value of allelic richness per locus and per population with FSTAT version 2.9.3.2 (Goudet 1995) and calculated the number of private alleles in each population (i.e., the number of alleles specific to a population), because allelic richness can be standardized independently of sample size, and both measures are independent of heterozygosity. The allelic richness was computed as in El Mousadik and Petit (1996), that is, a mean number of alleles for each locus in each population, with populations weighted in inverse proportion to their sample sizes in order to give the same weight to all populations. As FSTAT is not able to handle haploid and diploid data in the same analysis, we merged the haploid data from pairs of males to create “false diploids” without changing allelic frequencies. We then analyzed all “diploids” in a single run.

#### Statistical analysis

We used a hierarchical generalized linear model (binomial error and logit link) to assess the effect of population structure on the proportion of diploid males (DMP). Population structure was characterized by different nested explanatory variables: *Allelic richness* (All\_rich) and *number of private alleles* (Priv\_all) nested in the *habitat type* (Habit), that is, mainland, island, or captive (see Table 1). Data were all analyzed with the statistical software R (version 3.2.2, R Core Team, 2015). The significance of terms in the statistical model was assessed with type II sum-of-

**Table 2.** Characteristics of two multiplex PCRs amplifying 19 microsatellite loci in *Venturia canescens*.

Primer sequences 5'–3'								
Multiplex	Locus	Repeat motif	F-Primer	R-Primer	F 5' label	Concentration (μmol/L)	Size range (bp)	GenBank no.
I	VC068	(GA) <sub>9</sub>	TATCCTTCCAGCATTCGTCC	CTCGCTCGGTGGAACACTAC	FAM	0.1	104–120	KX258853
	Vcan071	(CAA) <sub>11</sub>	CTCTACGCACCTCCCTTCAC	TGTACGTTGGCACTTGAGC	FAM	0.1	223–254	GU053679
	VC092	(AG) <sub>9</sub>	TGTTGGCTCTTGTGTAAGT	CTCTCGTCAATTGCGTCTGT	FAM	0.1	284–307	KX258854
	VC094	(GT) <sub>8</sub>	TCGATTGCTTGAATCCTCTG	CACATAATTTCCCTTGACAC	FAM	0.2	429–436	KX258855
	VC009	(CAA) <sub>26</sub>	AACAGCAACAGCAACAGGTG	ACTTTGCCACGTGATTTC	VIC	0.1	313–337	KX258847
	VC036	(TTC) <sub>12</sub>	GTCAGCGATACACGCACG	GTACGCTCTTATTCTCGCG	NED	0.1	226–254	KX258849
	VC002	(AG) <sub>11</sub>	TCCGTTTCGTCTCATTTATAATCA	ATGATTGCTCTGACCGCTTC	NED	0.4	324–342	KX258845
	VC001	(AG) <sub>10</sub>	TTTCGCCAGTTTGTCTGAAG	AACGAAACGAAATTTACAAATCG	NED	0.4	391–447	KX258844
	VC066	(CAA) <sub>7</sub>	ACACATTTGAACCTCGAATCGAA	TCCTCTTGAAGCTCAAATTCG	PET	0.2	87–90	KX258852
	VC060	(CTT) <sub>12</sub>	TATCTCGCGTTCTATTCCGG	AGGCGCTGATTCGAAGTTAA	PET	0.2	206–231	KX258851
	VC106	(AG) <sub>11</sub>	CAAGCATGTATGTGATCGGTG	CGTAACATTTTCGCGTTGGC	FAM	0.2	89–97	KX258856
	Vcan073	(TGT) <sub>15</sub>	GGTCCAACGGTACTTCTCTGA	ACTTCGTCAGCCCTACCTT	FAM	0.2	227–267	GU053681
II	VC047	(AG) <sub>11</sub>	ACCTGAGGGCACTATCTGTT	C GAAAGTTAATTTCTAGACC GAGC	VIC	0.2	141–157	KX258850
	VC031	(TC) <sub>11</sub>	TCAGTCACTTAGTGCACTTGGAA	GGGTGGTGTAAATAGAGCGAGG	VIC	0.2	227–261	KX258848
	VC006	(AG) <sub>8</sub>	GACTAATGCAAGGAGGTTGTCG	GGCACAGTTTATGTTTCAGCG	VIC	0.2	317–357	KX258846
	VC120	CCCT(CCCCT) <sub>2</sub>	CAATCGATCAACGATACATTCG	GCAGGGTAGCAGCTTAGTGG	NED	0.2	92–128	KX258858
		(CT) <sub>2</sub> CCCT(CT) <sub>9</sub>						
	Vcan106	(TC) <sub>24</sub>	CCTCATCTCGAGGGAGGATT	ATCGCGAGTTGCGTAGTTTC	NED	0.2	183–222	GU053714
	VC107	(CAG) <sub>5</sub> (CAA) <sub>12</sub>	CAACATCACCAACAACACCA	CACCTGCACATGTCGTTGC	PET	0.2	85–108	KX258857
		CAGAGG(TA) <sub>2</sub>						
	Vcan088	(CA) <sub>44</sub>	AGTAACCGGTGAGCCCTTTGG	CAGTTCCAATTTCCACACA	PET	0.2	133–150	GU053696

All the microsatellites were developed in this study, except Vcan071, Vcan073, Vcan106, and Vcan088 that come from Mateo Leach *et al.* (2012).

squares tested with analyses of deviance with the likelihood ratio test based on the  $\chi^2$  distribution (*car* package (Fox and Weisberg 2010)). We used nonparametric analysis to test for population differentiation (Kruskal–Wallis test on allelic richness and the number of private alleles). Due to the low sample size (11 when using populations and three when testing the effect of habitat types) and therefore the lack of statistical power, we estimated the effect size by computing the Cohen's *d* (Cohen 1977; Nakagawa and Cuthill 2007) with *effsize* library in R (Torchiano 2015) and used the threshold provided in Cohen (1992) to assess the magnitude of the effect, that is,  $|d| < 0.2$ : negligible,  $|d| < 0.5$ : small,  $|d| < 0.8$ : medium, otherwise large.

In all multiple analyses such as tests for HW equilibrium, *P*-values were corrected for multiple testing via the false discovery rate (FDR) procedure (Benjamini and Hochberg 1995). Means are thereafter presented with standard errors unless indicated otherwise.

## Results

### Insect sampling and microsatellite markers

A total of 108 females were collected between 2010 and 2013, and 627 males were collected in 11 populations

(Table 1 and Fig. 1). No males were found in the French, Italian, Greek, Malta, and Cyprus islands, and as only one to three females were sampled in these locations, they were not used for the microsatellite development. Therefore, we used 75 females from Nice and Valence (2010) for the development of genetic markers.

### Validation of microsatellite markers for population genetic studies

For all the nineteen microsatellites developed, amplification was obtained for all the arrhenotokous *V. canescens* females trapped in Nice (Nice10 population) and in Valence (Val10 populations). The number of alleles ranged from 2 to 14 (mean number of alleles =  $7.95 \pm 0.76$ , Table 3). The two multiplex PCRs displayed a similar range (multiplex I: 2–12 alleles, multiplex II: 4–14 alleles) and mean number of alleles (multiplex I:  $7.3 \pm 0.92$ , multiplex II:  $8.67 \pm 1.25$ , *t*-test:  $t = -0.88$ , *df* = 15, *P* = 0.39). The markers had a maximum frequency of null alleles equal to 8.5% (Vcan073 locus, Val10 population; mean:  $1.7 \pm 0.6\%$  in Nice10 population and  $1.8 \pm 0.7\%$  in Val10 population) and a null median frequency for both populations. No departure from the HW equilibrium was found in any of the populations

**Table 3.** Genetic diversity at 19 microsatellite loci for natural populations of *Venturia canescens* sampled in 2010 near Nice and Valence, south-east of France.

Multiplex	Primer name	Number of alleles	Population							
			Nice10 ( <i>n</i> = 44)				Val10 ( <i>n</i> = 37)			
			<i>H<sub>e</sub></i>	<i>H<sub>o</sub></i>	HW <i>P</i> -value	Null allele frequency	<i>H<sub>e</sub></i>	<i>H<sub>o</sub></i>	HW <i>P</i> -value	Null allele frequency
I	VC068	7	0.742	0.727	0.848	0.012	0.826	0.865	0.771	0.000
	Vcan071	11	0.809	0.750	0.493	0.036	0.798	0.838	0.728	0.000
	VC092	7	0.636	0.705	0.390	0.000	0.600	0.676	1.000	0.000
	VC094	4	0.592	0.545	0.771	0.034	0.511	0.541	0.813	0.000
	VC009	8	0.697	0.864	0.135	0.000	0.649	0.703	1.000	0.000
	VC036	7	0.540	0.568	0.771	0.000	0.450	0.432	0.771	0.000
	VC002	7	0.577	0.591	0.813	0.026	0.716	0.757	0.771	0.000
	VC001	12	0.600	0.477	0.370	0.090	0.655	0.595	0.176	0.046
	VC066	2	0.487	0.386	0.509	0.069	0.491	0.486	1.000	0.003
II	VC060	8	0.549	0.591	0.927	0.000	0.558	0.568	0.135	0.011
	VC106	4	0.711	0.750	0.976	0.000	0.728	0.676	0.307	0.019
	Vcan73	10	0.672	0.818	0.746	0.000	0.706	0.541	0.135	0.092
	VC047	5	0.573	0.636	0.821	0.000	0.607	0.541	0.396	0.013
	VC031	14	0.650	0.591	0.390	0.048	0.674	0.595	0.493	0.083
	VC006	11	0.673	0.773	0.813	0.000	0.593	0.595	0.771	0.000
	VC120	7	0.515	0.500	0.771	0.000	0.593	0.459	0.135	0.059
	Vcan106	14	0.875	0.886	0.400	0.008	0.836	0.919	0.746	0.000
	VC107	6	0.724	0.705	0.493	0.000	0.603	0.622	0.479	0.022
	Vcan088	7	0.644	0.682	0.813	0.009	0.489	0.541	0.821	0.000

*n*: number of females analyzed; *H<sub>e</sub>*: expected heterozygosity; *H<sub>o</sub>*: observed heterozygosity; HW *P*-value: *P*-value of the test for Hardy–Weinberg equilibrium after FDR correction.



(Table 3), and the observed heterozygosity ( $H_o$ ) was similar in the two multiplex PCRs in both populations (Mean  $H_o$  multiplex I:  $0.63 \pm 0.03$ , multiplex II:  $0.66 \pm 0.03$ ;  $t$ -test:  $t = -0.54$ ,  $df = 36$ ,  $P = 0.59$ ; Table 3). Two pairs of microsatellites were detected as linked when both populations were considered: Vcan073/Vcan106 ( $\chi^2 = \infty$ ,  $df = 4$ ,  $P < 0.0001$ ) and VC107/Vcan106 ( $\chi^2 = 24.2$ ,  $df = 4$ ,  $P = 0.0063$ ). However, when populations were analyzed separately, the pairs of loci were linked only in one population (Vcan073/Vcan106 in Val10 and VC107/Vcan106 in Nice 10), we therefore decided to keep all loci. Therefore, the two multiplex PCR can be used for genetic studies with equivalent performance.

### Validation of markers for ploidy assessment

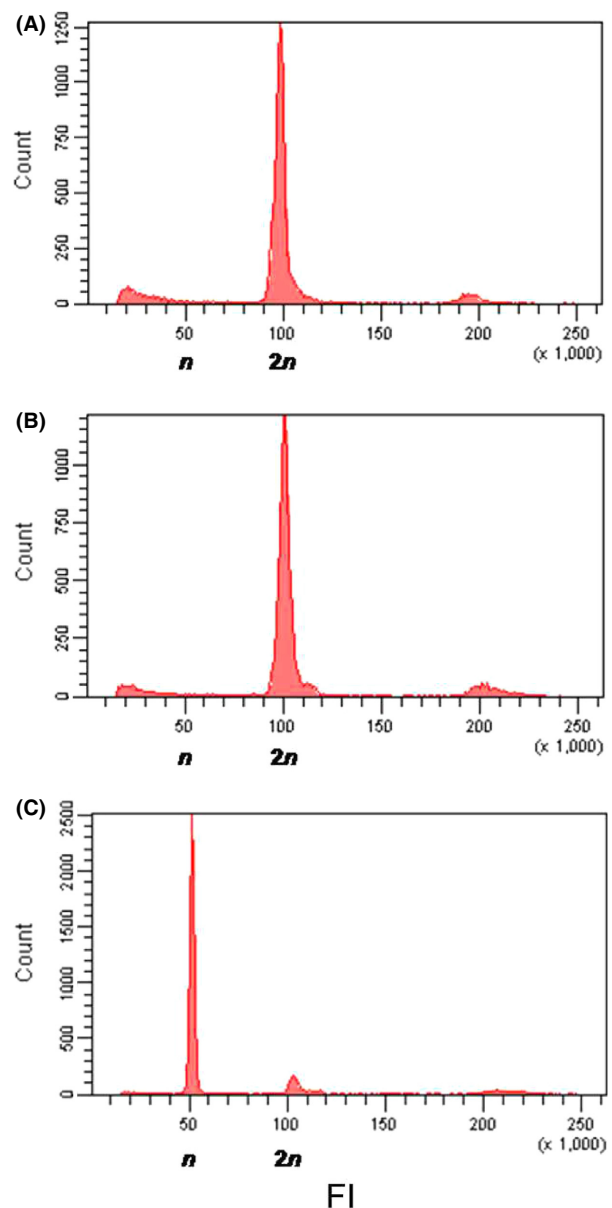
The probability that a diploid male produced by sibmating is homozygous for all microsatellite loci of the multiplex I was low enough ( $P = 0.0023$ ) to rely on microsatellites for ploidy assessment. This was confirmed by the congruence between flow cytometry and microsatellite genotyping results. Haploid and diploid males were easily discriminated by flow cytometry, with diploid males presenting a profile similar to that of diploid females (Fig. 2). For the 39 males tested, the ploidy measured by flow cytometry and genotyping analyses matched perfectly.

### Population genetic structure and proportion of diploid males

After genotyping with the markers of multiplex I, we discarded 28 males for which more than one loci did not amplify. As a result, 599 males (95.5%) were successfully genotyped and used in the analysis.

### Population differentiation

The population structure was estimated through different parameters such as allelic richness (All\_Rich) and the number of private alleles (Priv\_all) from male genotyping data (Table 4). The number of private alleles was marginally significantly different between habitat types (mainland, island, and captive, Kruskal–Wallis test,  $\chi^2 = 5.68$ ,  $df = 2$ ,  $P = 0.058$ ), and allelic richness significantly differed between habitat types (Kruskal–Wallis test,  $\chi^2 = 6.72$ ,  $df = 2$ ,  $P = 0.035$ ) and populations (Kruskal–Wallis test,  $\chi^2 = 18.51$ ,  $df = 10$ ,  $P = 0.047$ ). Both measures shared the same trend suggesting that the diversity is the lowest in captive populations and the highest in mainland (Table 4). Effect sizes were medium or large for each comparison for both variables (mainland–island, All\_Rich:  $d = 2.35$ , Priv\_all:  $d = 1.09$ ; mainland–captive,



**Figure 2.** Flow cytometric histograms of the number of nuclei registered as a function of their fluorescence intensity (FI), for a representative female (A), diploid male (B), and haploid male (C). FI is expressed in an arbitrary unit calibrated to value 100 at the fluorescence intensity with the highest number of nuclei registered in females, which are known to be diploid.

All\_Rich:  $d = 1.56$ , Priv\_all:  $d = 1.52$ ; island–captive, All\_Rich:  $d = 0.67$ , Priv\_all:  $d = 1.41$ ). The sampling year had no impact on the mean allelic richness for populations sampled over 2 years ( $t$ -test, Nice11–Nice13:  $t = -0.247$ ,  $df = 15.9$ ,  $P = 0.808$  and Mlc12–Mlc13:  $t = -0.124$ ,  $df = 15.9$ ,  $P = 0.903$ ). Allelic richness of the captive population from Israel (CapIsr) appeared different

**Table 4.** Characteristics of *Venturia canescens* populations based on the analysis of males.

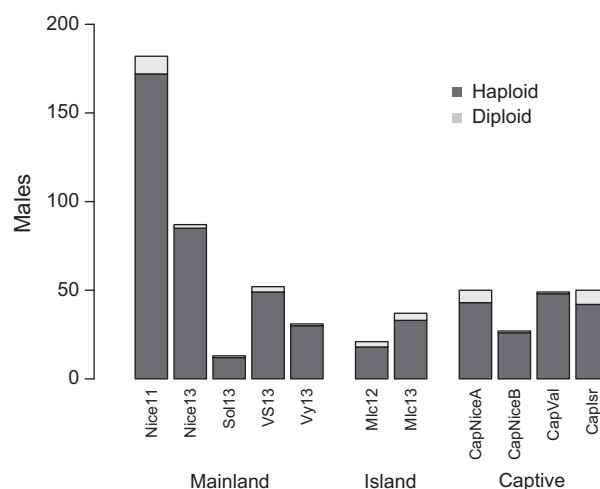
Location	Population	Number of sampled males	Number of genotyped haploid males	Number of genotyped diploid males	Mean number of alleles	Overall allelic richness	Number of private alleles
Mainland							
France	Nice11	190	172	10	6.78 ± 0.92	3.37	8
	Nice13	90	85	2	6.00 ± 0.75	3.52	0
	Sol13	16	12	1	3.78 ± 0.43	3.58	2
Spain	VS13	58	49	3	5.67 ± 0.78	3.41	3
	Vy13	33	30	1	5.22 ± 0.64	3.68	4
Mean Mainland		77.4 ± 3.5	69.6 ± 3.4	3.4 ± 0.9	5.49 ± 0.34	3.51 ± 0.14	3.4
Island							
Spain	Mlc12	21	18	3	4.22 ± 0.43	3.27	0
	Mlc13	38	33	4	4.56 ± 0.47	3.25	1
Mean Island		29.5 ± 1.6	25.5 ± 1.5	3.5 ± 0.3	4.39 ± 0.31	3.26 ± 0.18	0.5
Captive							
France	CapNiceA	50	43	7	4.33 ± 0.37	3.32	0
	CapNiceB	31	26	1	3.78 ± 0.52	3.04	0
	CapVal	50	48	1	3.67 ± 0.44	3.08	0
Israël	CapIsr	50	42	8	2.44 ± 0.24	2.05	0
Mean Captive		45.3 ± 0.7	39.8 ± 0.8	4.3 ± 0.9	3.56 ± 0.23	2.87 ± 0.15	0

Allelic richness and mean number of alleles were computed with FSTAT software. This software being unable to handle both haploid and diploid data in the same analysis, the co-occurrence of haploid and diploid males constrained us to merge pairs of haploid data to create “false diploid” males. The results presented are mean ± SE.

from all the other populations except the captive populations from Nice and Valence (CapNiceB and CapVal, pairwise *t*-tests between populations with FDR correction for multiple testing, data not shown). As the CapIsr population was highly differentiated from the other populations, it could potentially drive the significant trend observed. Indeed, when allelic richness was compared without CapIsr, the difference between populations disappeared (Kruskal–Wallis test,  $\chi^2 = 4.745$ , *df* = 9, *P* = 0.856), and the difference between habitat types became only marginally significant (Kruskal–Wallis test,  $\chi^2 = 5.804$ , *df* = 2, *P* = 0.055).

### Diploid male proportion and genetic diversity

Diploid males were found in all populations, but in variable proportions (from DMP = 0.02 ± 0.02 in CapVal to DMP = 0.16 ± 0.05 in CapIsr; Table 4 and Fig. 3). The sampling year had no impact on the proportion of diploid males for populations sampled over 2 years (proportion test, Nice11–Nice13:  $\chi^2 = 0.760$ , *df* = 1, *P* = 0.383 and Mlc12–Mlc13:  $\chi^2 = 5.10^{-32}$ , *df* = 1, *P* = 1). As expected, the DMP was affected by habitat type (LR  $\chi^2 = 7.07$ , *df* = 2, *P* = 0.029, Table 5), with a negative relationship between the DMP and the allelic richness of each habitat types (Fig. 4). When removing the CapIsr population, the effect of the habitat type became non-significant (*P* = 0.105), but we still found a negative influence of allelic richness (nested within habitat types) on DMP (LR  $\chi^2 = 6.80$ , *df* = 2, *P* = 0.033, Table 5).

**Figure 3.** Number of haploid and diploid males in the 11 populations within each habitat type.

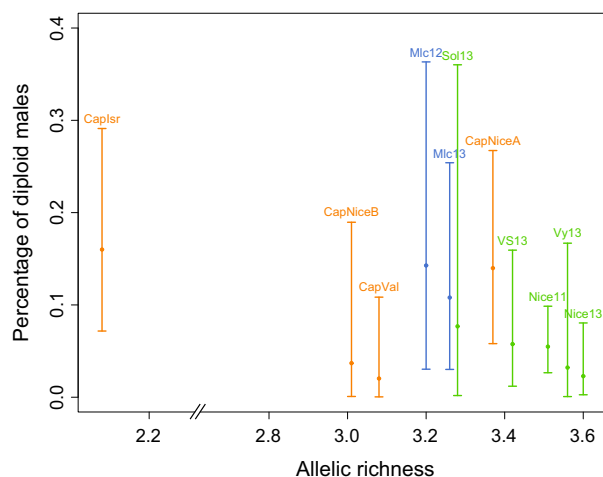
### Discussion

We developed highly variable microsatellite markers for the parasitoid wasp *V. canescens*. Polymorphism, HW equilibrium, the absence of linkage disequilibrium, and negligible null allele frequencies make these markers suitable for population genetic studies. In addition, relatively high rates of heterozygosity enable a reliable measure of male ploidy, confirmed by flow cytometry. Using these markers, we estimated within-population genetic diversity and the proportion of diploid males in eleven locations

**Table 5.** Effect of habitat type and genetic diversity on diploid male proportion.

Response variable	Diploid male proportion		
	LR $\chi^2$	df	P
With Caplsr population			
Habit	7.0710	2	<b>0.029</b>
Habit:All_rich	2.4942	2	0.287
Habit:Priv_all	0.5125	1	0.474
Without Caplsr population			
Habit	4.5108	2	0.104
Habit:All_rich	6.7997	2	<b>0.033</b>
Habit:Priv_all	0.5125	1	0.474

LR, Likelihood ratio; df, degrees of freedom; Habit, habitat type; All\_rich, allelic richness; Priv\_all, private alleles.  $P < 0.05$  highlighted in bold.

**Figure 4.** Percentage of diploid males according to allelic richness in the 11 populations, with 95% confidence intervals. Colors represent the three habitat types of locations: light green for the mainland populations, light blue for the island populations, and orange for the captive populations.

around the Mediterranean Sea, as well as in captive populations. We found a tendency for genetic diversity to be reduced in small and isolated populations, and the proportion of diploid males to increase with decreasing genetic diversity. These patterns are expected theoretically in a scenario whereby reduced population size yields decreased genetic diversity (and decreased allelic polymorphism at the sl-CSD locus), increased proportion of diploid males, decreased population growth rate, and, if dramatic enough, population extinction (a scenario referred to as the diploid male vortex; Zayed and Packer 2005). Hence, our results suggest that habitat type and population history are key features that should be considered when studying genetic diversity in parasitoids with sl-CSD.

The effect of habitat type on genetic diversity was marked by a lower diversity in captive populations. These bottlenecked and completely isolated populations were characterized by a lower allelic richness compared to either island or mainland populations, and a lower number of private alleles compared to mainland populations (Table 3). Because the number of private alleles is highly dependent on sampling effort, the effect of habitat type on the number of private alleles could simply result from the high number of individuals captured in the mainland population of Nice. Besides, the captive population from Israel was highly differentiated from all populations as a result of a very small foundress number (11 females). This population had the lowest allelic richness and mean number of alleles, and hence, it is likely that these extreme values had driven the effect of habitat type. Indeed, the population effect disappeared when discarding the population from Israel. Nonetheless, the effect of habitat type remained marginally significant, suggesting that the trend of higher allelic richness in mainland compared to island and captive populations is indeed real.

The decrease in genetic diversity co-occurring with higher rates of inbreeding in small and isolated populations, such as island populations, has been documented in various species (mammals, birds, fishes, insect or plants, Frankham 1997; Furlan *et al.* 2012). In *V. canescens*, the higher genetic diversity in mainland compared to captive populations could be explained by efficient dispersal. In this species, adults are good dispersers (Desouhant *et al.* 2003), a feature that corroborates the absence of genetic structure at the scale of southeastern France (Schneider *et al.* 2002). In island and captive populations, founder effect and genetic drift combined with constrained dispersal should lead to genetic erosion, a phenomenon that is also observed in other species of the order Hymenoptera such as bumblebees or orchid bees (Ellis *et al.* 2006; Schmid-Hempel *et al.* 2007; Boff *et al.* 2014).

Consistent with these effects of habitat type on diversity at microsatellite loci, the proportion of diploid males was affected by the habitat type with more frequent diploid males in island and captive populations than in mainland locations. The highest proportion (16%) was observed in the captive population from Israel, which is congruent with the lowest genetic diversity estimated. In other species, diploid male production was also found to be higher in isolated population compared to larger genetically diverse populations (Kukuk and May 1990). Overall, the proportion of diploid males in *V. canescens* (6.8% overall, CI 95% [5.0–9.2%]) is in the order of magnitude of estimations in other species of parasitoids with sl-CSD: 10% in a native population of *C. glomerata* (Ruf *et al.* 2013) and 15% in a population of *C. rubecula* introduced for biological control (de Boer *et al.* 2012).

It is however important to notice that despite a high sampling effort in several islands, we caught males only on one island (Mallorca), for two consecutive years. It is therefore possible that the trend of lower genetic diversity and higher proportion of diploid males compared to mainland populations are due to a peculiar composition specific to Mallorca population, and rather than an effect of the habitat type “island” and its isolation. The habitat isolation effect is nonetheless corroborated by the genetic erosion observed in captive populations, and thus likely to be real, but would require new samplings in other islands to be confirmed.

Behaviors such as natal dispersal and mate choice can mitigate the effects of decreased genetic diversity and hence attenuate diploid male production. In *C. glomerata*, a biased fertilization occurs and limits genetic incompatibility, leading to a DMP lower than expected in field populations (Ruf *et al.* 2013). On the contrary, the lack of mate discrimination in *C. rubecula* could explain the relatively high DMP observed in its field populations (de Boer *et al.* 2012). Laboratory studies in *Bracon brevicornis* have reported an avoidance of mating with a partner harboring the same allele at the sl-CSD locus (i.e., avoidance of matched matings; Thiel *et al.* 2013), and sib-mating avoidance was observed in *V. canescens* (Metzger *et al.* 2010a). Such behaviors should reduce the DMP and thus lessens the production of unfit offspring (Parker 1983; Chuine *et al.* 2015).

The higher proportion of diploid males in captive and island populations of *V. canescens* could result from a lower genetic diversity in isolated populations, or a downgrade in discrimination of females against related males. In a number of species, females adapt future mate choice according to the genotype of their first mate [lizards (Laloi *et al.* 2011; Breedveld and Fitze 2015); beetles (Dowling *et al.* 2007); *Drosophila* (Chapman and Partridge 1998)]. This hypothesis may not hold for *V. canescens* because females are monoandrous (Metzger *et al.* 2010b). However, as island or captive populations are genetically eroded, the probability for a female to encounter a male bearing a similar allele at the *csd* locus is higher; possibly, successive encounters with low-quality males before mating could lead to an adaptive decline in sib-avoidance. This could explain the increase in diploid male production in small or isolated populations. At last, the presence of diploid males even in mainland populations where the genetic diversity is high and where no departure from HW equilibrium was detected could also be due to imperfect sib-mating avoidance, as observed under the laboratory conditions (Metzger *et al.* 2010a).

Our study suggests that diploid male production in insects of the order Hymenoptera reflects fitness decline resulting from inbreeding in small and isolated habitats.

Sex determination via sl-CSD imposes a major genetic constraint and may reflect an “unintelligent design” (*sensu* van Wilgenburg *et al.* 2006), which makes some hymenopterans highly sensitive to isolation. Hence, our study raises the question of population conservation (Zayed 2009) and highlights that small organisms such as insects may also suffer from habitat destruction and fragmentation.

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## Conflict of Interest

None declared.

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## Annex:

Calculation of the probability that a F2 male, produced by sibmating, is homozygous for all 10 markers from multiplex I.

A sample of 30 females was collected in a three- to five-generation-old captive population (founded in autumn 2009 with about 40 females from Nice). These females constitute the F0 generation and had presumably mated randomly in the population. Their offspring (generation F1) were constrained to mate between siblings (Fig. A1). We computed the probability that an F2 male, produced via sibmating, is homozygous for all genetic markers, using allelic frequencies and inbreeding coefficient ( $F_{is}$ ) of F0 females.

We first calculated the probability that an F2 diploid individual is homozygous at one microsatellite locus, given the genotypes of its grandparents (F0 generation) at this locus (Fig. A1). Then, for each locus  $k$ , we calculated the probability that an F0 female is heterozygous at this locus as  $P_k(\text{Het}_{F0}) = (1 - F_{is}) \times H_e$  and the probability that she is homozygous as  $P_k(\text{Hom}_{F0}) = 1 - P_k(\text{Het}_{F0})$ , where  $H_e$

is expected heterozygosity at loci  $k$  under HW equilibrium. We calculated as follows the conditional probabilities that the F0 male carries at loci  $k$  a different (DA) or common (CA) allele with the F0 female, knowing that the female is heterozygous ( $\text{Het}_{F0}$ ) or homozygous ( $\text{Hom}_{F0}$ ):

$$P_k(\text{CA}|\text{Het}_{F0}) = \sum_{i=1}^n \sum_{\substack{j=2 \\ i < j}}^n \left( \frac{2p_i p_j}{H_e} \right) (p_i + p_j)$$

$$P_k(\text{DA}|\text{Het}_{F0}) = 1 - P_k(\text{CA}|\text{Het}_{F0})$$

$$P_k(\text{CA}|\text{Hom}_{F0}) = \sum_{i=1}^n \left( \frac{p_i^2}{1 - H_e} \right) p_i$$

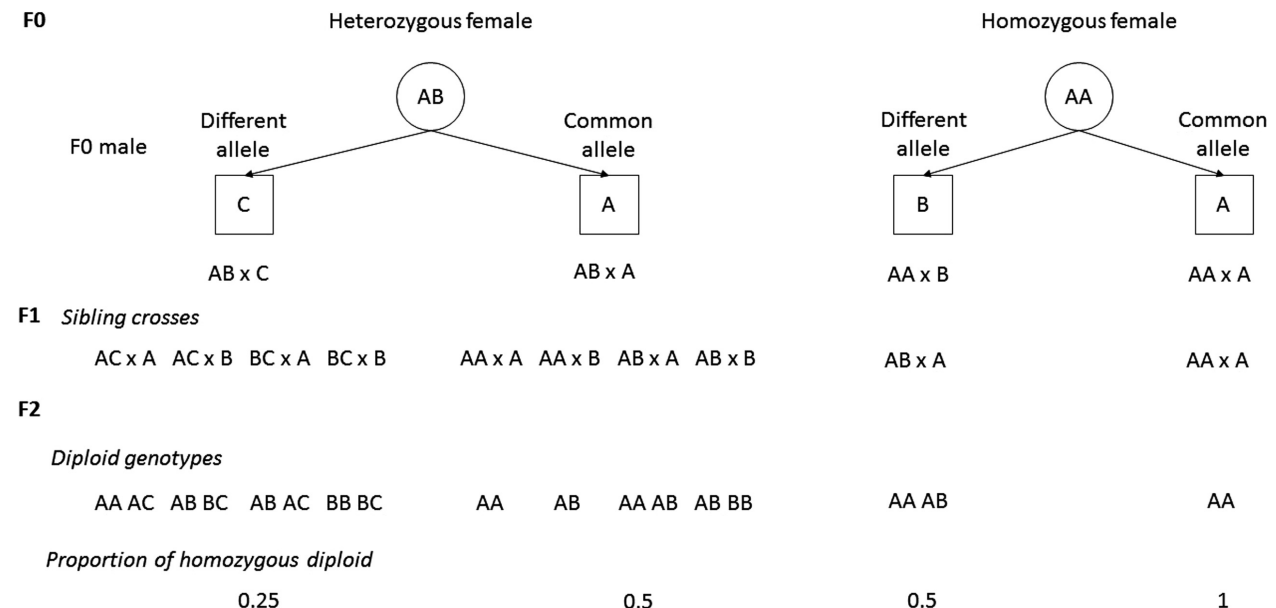
$$P_k(\text{DA}|\text{Hom}_{F0}) = 1 - P_k(\text{CA}|\text{Hom}_{F0})$$

$p_i$  and  $p_j$  are the frequencies of alleles  $i$  and  $j$ , respectively. Using the proportions of F2 homozygous from Figure A1, the probability that an F2 diploid is homozygous as locus  $k$  was as follows:

$$P_k(\text{Hom}_{F2}) = P_k(\text{Het}_{F0}) (0.5P_k(\text{CA}|\text{Het}_{F0}) + 0.25P_k(\text{DA}|\text{Het}_{F0})) + P_k(\text{Hom}_{F0}) (P_k(\text{CA}|\text{Hom}_{F0}) + 0.5P_k(\text{DA}|\text{Hom}_{F0}))$$

The probability that a diploid F2 is homozygous at all 10 loci from multiplex I was as follows:

$$P(\text{Hom}_{F2}) = \prod_{k=1}^{10} P_k(\text{Hom}_{F2}).$$



**Figure A1.** Proportion of heterozygous diploid F2 at one microsatellite locus, according to the genotype of the F0 pair.